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Topical Review

Ca^{2+} Signalling in Brain Synaptosomes Activated by Dinucleotides

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Abstract. Diadenosine polyphosphates are a family of dinucleotides formed by two adenosines joined by a variable number of phosphates. Diadenosine tetraphosphate, Ap_4A, diadenosine pentaphosphate Ap_5A, and diadenosine hexaphosphate, Ap_6A, are stored in synaptic vesicles and are released upon nerve terminal depolarization. At the extracellular level, diadenosine polyphosphates can stimulate presynaptic dinucleotide receptors. Responses to diadenosine polyphosphates have been described in isolated synaptic terminals (synaptosomes) from several brain areas in different animal species, including man. Dinucleotide receptors are ligand-operated ion channels that allow the influx of cations into the terminals. These cations reach a threshold for N- and P/Q-type voltage-dependent calcium channels, which become activated. The activation of the dinucleotide receptor together with the activation of these calcium channels triggers the release of neurotransmitters. The ability of Ap_5A to promote glutamate, GABA or acetylcholine release has been recently described by the present authors in rat midbrain synaptosomes.

Key words: Ap_4A — Ap_5A — Diadenosine polyphosphates — Purinergic receptors — Voltage dependent calcium channels

Introduction

Brief Historical Background

Dinucleoside polyphosphates comprise a family of nucleotides formed by two adenosine moieties linked by a variable phosphate chain. These dinucleotides, characterized by a 5′, 5′ bridge between the two nucleosides, have been known to exist since 1953, when diadenosine pyrophosphate (Ap_2A) and diuridine pyrophosphate (Up_2U) were detected during the synthesis of the compound Ap_5U (Christie et al., 1953). However, it was not until 1963 that these compounds were ascribed a biological role, when Gp_4G and Gp_3G were identified in the encysted embryos of the brine shrimp *Artemia* and *Daphnia* (Finamore & Wagner, 1963). The first time adenylated dinucleoside polyphosphates were observed in vivo was in 1966, when Ap_4A was detected, along with other nucleotidic compounds, in a reaction, whereby AMP from an aminoacyladenylate is donated to ATP, catalyzed by some aminoacyl-tRNA synthetases (Zamecnik et al., 1966). Since then, diadenosine polyphosphates have been recognized as being physiologically relevant molecules involved in processes as essential as the control of cell proliferative state (Rapaport & Zamecnik, 1976), initiation of DNA replication (Grummt, 1978) or binding to DNA polymerase-α (Baril et al., 1983). A detailed compendium on the chemical and biochemical properties of these compounds may be found in an exhaustive monograph edited by McLennan (1992).

In a more recent setting, it was demonstrated that the product of the human FHIT tumour suppressor gene, a protein termed Fhit, shows Ap_3A hydrolase activity (Barnes et al., 1996; Brenner et al., 1999). This finding is of extreme interest, since a lack of Fhit is associated with cancer in many tissues, and the re-expression of this protein leads to programmed cell death (Huebner et al., 1999). If the correct functioning of this protein depends on Ap_nA binding, all mechanisms whereby these dinucleotides are synthesized may be considered upstream regulators of Fhit (for a full review, *see* McLennan et al., 2001). Another feature of interest is the presence of diadenosine polyphosphates in the secretory vesicles of secretory cells and neurons, and their consequent release after

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stimulation. The first evidence for this emerged from studies on chromaffin cells, in which Ap_4A, Ap_5A, and later on Ap_6A, were found to be co-stored with catecholamines and ATP within chromaffin granules (Rodriguez del Castillo et al., 1988; Pintor et al., 1992b). Later, these dinucleotides were identified in rat brain synaptic terminals, presumably co-stored with other classical transmitters (Pintor et al., 1992a).

In parallel to the discovery of the important role played by diadenosine polyphosphates in the nervous system, it was also found they had close links with the cardiovascular system. Besides their presence, a physiological role in controlling vascular tone and platelet aggregation was described for these dinucleotides (Harrison, Brossmet & Goody, 1975; Flodgaard & Klenow, 1982; Lütjhe & Ogilvie, 1983, 1984; Schlüter et al., 1994; Hilderman & Christensen, 1998). The synthesis of new dinucleotide analogues with powerful antiplatelet aggregation activities has been recently reported (Walkowiak et al., 2002).

Diadenosine Polyphosphates in the Nervous System

Storage in Secretory Granules and Vesicles

As previously indicated, chromaffin cells were the first neural site found to contain diadenosine polyphosphates (Rodriguez del Castillo et al., 1988). Chromaffin cells store dinucleotides within chromaffin granules by means of a nucleotide transporter with mnemonic properties that also transport mononucleotides (Gualix et al., 1997). This system allows storage of Ap_4A, Ap_5A and Ap_6A in chromaffin cells at concentrations of 1.47 nmol/10^6 cells, 1.53 nmol/10^6 cells and 1.46 nmol/10^6 cells, respectively (Rodriguez del Castillo et al., 1988; Pintor et al., 1992b).

The presence of Ap_4A, Ap_5A and Ap_6A in the synaptic vesicles of rat brain synaptic terminals was demonstrated by HPLC analysis (Pintor et al., 1992a). These molecules are transported to these vesicles by a system with features similar to those observed in chromaffin cells (Gualix, Pintor & Miras-Portugal, 1999). The concentrations of these nucleotides in synaptic terminals in pmol per mg of protein are 169, 159 and 100 for Ap_4A, Ap_5A and Ap_6A, respectively (Pintor et al., 1992a).

Diadenosine Polyphosphate Release in Neural Models

Chromaffin cells are able to release diadenosine polyphosphates when challenged with nicotinic agonists such as carbamylcholine (carbachol). When these cells were stimulated with 100 μM carbachol, they released 11.1 nmol $Ap_4A/10^6$ cells and 15.8 nmol $Ap_5A/10^6$ cells (Pintor, Torres & Miras-Portugal, 1991b). Although it is difficult to calculate the extracellular concentration of these dinucleotides, an approximation can be made as follows: if the extracellular volume into which the dinucleotides are released is equal to that calculated for a single chromaffin cell, their concentration in the extracellular milieu would be roughly 27 μM (Pintor et al., 1991a).

It is possible to determine the amounts of these dinucleotides released from rat brain synaptic terminals in response to 4-aminopyridine and veratridine. Concentrations of 14.57 and 11.64 pmol/mg protein were recorded for Ap_4A and Ap_5A in response to 4-aminopyridine, and corresponding values of 19.93 and 16.62 pmol/mg protein in response to veratridine (Pintor et al., 1992a). The real extracellular concentrations of these diadenosine polyphosphates cannot be established since synaptic terminals are heterogeneous in size. Nevertheless, using the push-pull technique, it has been possible to evaluate the levels of these compounds in the live rat before and after amphetamine stimulation.

Up until now, the push-pull method was the only known approach to quantifying extracellular levels of diadenosine polyphosphates in the CNS. By cannulating the caudate putamen in the conscious rat, we were able to establish that Ap_4A and Ap_5A levels in the absence of any external stimulus are below 2 nM (Pintor et al., 1993). Stimulation of the caudate putamen with amphetamine sulphate increased the motor activity of the rat along with Ap_nA release. Following stimulation, Ap_4A and Ap_5A levels were 12.9 and 11.5 pmol/200 μL, respectively, corresponding to concentrations of 64.5 and 57.5 nM (Pintor et al., 1995). It should be noted that the release of nucleotides occurs via a Ca^{2+}-dependent mechanism, though in the case of amphetamine-induced release of these dinucleotides, the mechanism is more complex. Amphetamine provokes the non-exocytotic release of catecholamines (mainly dopamine). In turn, these catecholamines can stimulate other terminals that release dinucleotides in a calcium dependent fashion (Pintor et al., 1995).

Specific Receptors for Diadenosine Polyphosphates in the CNS: The Dinucleotide Receptor

Most of the extracellular actions of diadenosine polyphosphates can be attributed to their interaction with receptors that are also activated by ATP and other nucleotides. The effects of diadenosine polyphosphates on both ionotropic (P2X) and metabotropic (P2Y) ATP receptors have been described for a wide variety of tissues as well as on heterogeneously expressed receptors (Lazarowski et al., 1995; Communi et al., 1996; Pintor et al., 1996, 2000; Schachter et al., 1996; Wildman et al., 1999). Thus, the crucial physiological/pharmacological question regarding

the role of these dinucleotides in the CNS that needs to be addressed is: are there specific receptors for diadenosine polyphosphates different from ATP receptors?

Hilderman and coworkers (1991) showed the presence of saturable high-affinity binding sites for diadenosine polyphosphates in membrane preparations from mouse brain. Interestingly, these sites appeared to be specific for dinucleotides, since ATP was unable to displace the binding of radiolabelled Ap_4A.

In 1995, Pintor and Miras-Portugal showed that synaptic terminals isolated from rat midbrain responded to the addition of ATP and diadenosine polyphosphates by increasing the intrasynaptosomal calcium concentration. This was the first demonstration of the presence of presynaptic receptors for both compounds in the CNS. The effect of diadenosine polyphosphates appeared to be mediated by receptors different from those for ATP, indicated by a lack of cross-desensitization when these compounds were consecutively applied, though each agonist was able to desensitize itself. Additional proof for the presence of independent presynaptic receptors for ATP and Ap_nA was provided by the selectivity shown by the P2 antagonists PPADS and suramin. These compounds did not affect the Ca^{2+} increase evoked by diadenosine polyphosphates in rat synaptosomes but dramatically blocked Ca^{2+} entry induced by ATP and its synthetic analogues (Pintor & Miras-Portugal, 1995). In 1997, a new series of dinucleotides denoted diinosine polyphosphates were synthesized by enzymatic deamination of the corresponding diadenosine polyphosphate (Pintor, Gualix & Miras-Portugal, 1997a). These substances showed no agonistic effect on rat synaptosome receptors, but were able to effectively antagonize responses induced by Ap_nA. Diinosine pentaphosphate, or Ip_5I, has since then proved to be the most potent of theses substances, capable of blocking responses to Ap_nA at concentrations in the nanomolar range (IC_{50} = 4 nM). In contrast, micromolar concentrations of the antagonist were required to abolish ATP-elicited responses in the same preparation (Pintor et al., 1997a). Taken together, these differences between the actions of Ap_nA compounds and ATP and the inability of methylxanthines to block dinucleotide actions (excluding the participation of adenosine receptors), strongly suggested there are specific receptors for diadenosine polyphosphates in rat midbrain synaptic terminals. Here the term "specific" is taken to mean not sensitive to mononucleotides (ATP, UTP), adenosine, or their respective pharmacological analogues and sensitive only to dinucleoside oligophosphates. Accordingly, being selectively antagonized by nanomolar concentrations of Ip_5I, this receptor cannot be blocked by the classical P1 or P2 antagonists.

In the following years there was some discrepancy over the nomenclature to be adopted for this new type of receptor and several terms (P_{2D}, $P2Y_{APnA}$, P4, and dinucleotide receptor) have been used in the literature to describe both high-affinity binding sites or specific receptors for diadenosine polyphosphates. This imprecise nomenclature has led to some confusion on the identity of the presynaptic diadenosine polyphosphate receptor of the rat synaptosome preparation. According to its pharmacological properties, i.e., insensitivity towards mononucleotides and a divergent pharmacological profile that does not fit in with the P2 receptors described so far, and in the absence of any structural data, we propose it is not appropriate to define this receptor as a variant of the ATP receptor. Consequently, we prefer to use the term dinucleotide receptor over any other cited in the literature (Pintor & Miras-Portugal, 2000).

Properties of the Dinucleotide Receptor

There is some evidence to suggest the dinucleotide receptor is a receptor-operated Ca^{2+} channel. Among this evidence is its failure to respond to dinucleotides in the absence of extrasynaptosomal calcium, and its global functioning in the presence of G-protein modulators, which is not consistent with the idea that the receptor could be coupled to a channel via a G-protein mechanism (Pintor & Miras-Portugal, 1995; Pintor, Gualix & Miras-Portugal, 1997b). Nevertheless, the behavior of the receptor in the presence of non-hydrolizable GTP/GDP analogues can be effectively explained by activation of protein kinases that would, in turn, modulate the activity of the dinucleotide receptor (Pintor et al., 1997b; *see* following sections).

Using different dyes, it has been possible to establish whether this receptor is permeable to both Na^+ and Ca^{2+} or only to Ca^{2+}. Intracellular Ca^{2+} levels, as estimated by fura-2, and Na^+ levels, estimated by SBFI, indicate that diadenosine polyphosphate stimulation leads to Ca^{2+} entry, depolarizing terminals and opening voltage-dependent ion channels (Pintor & Miras-Portugal, 1995).

Connection with Voltage-dependent Ion Channels

After stimulating the synaptic terminal with adenine dinucleotides, Ca^{2+} entry through the dinucleotide receptor depolarizes the terminal and activates Na^+ and Ca^{2+} voltage-dependent channels. Na^+-channel activation was confirmed by treating the terminals with tetrodotoxin. The lack of Na^+ entry in the presence of the toxin was established by SBFI.

Several substances were used to selectively block L-, N-, P- or T-type voltage-gated Ca^{2+} channels. Among these only ω-conotoxin G-VI-A, which blocks N-type Ca^{2+} channels, was able to partially reduce the

Ca^{2+} entry induced by diadenosine polyphosphates. When Ca^{2+} entry in the presence of this toxin was explored further, an initial transient increase was revealed, suggesting the dinucleotide receptor elicits an initial Ca^{2+} entry that is voltage insensitive. Ca^{2+} entry was clearly diminished after this initial peak when ω-conotoxin G-VI-A was applied. This indicates the overall Ca^{2+} increase is really the sum of a voltage-independent transient rise, followed by a voltage-dependent increase mediated by an N-type Ca^{2+} channel. This was also observed when voltage-operated Ca^{2+} channels were inhibited by depolarization with 60 mM K^+. Under these conditions, the initial Ca^{2+} surge induced by Ap_5A remains unchanged but its subsequent entry via Ca^{2+} channels is abolished (Pintor & Miras-Portugal, 1995).

Presence of Dinucleotide Receptors in the Brain of Several Animal Species

Rat midbrain was the first tissue in which the dinucleotide receptor was characterized. However, subsequent studies have also demonstrated the presence of this receptor in synaptosomes preparations from the deermouse, guinea-pig and human brain. In all these models, the question of whether Ap_nA exerts its actions via ATP purinoceptors or through a separate population of receptors, has been addressed by cross desensitization experiments and studies based on antagonists (Pivorun & Nordone, 1996; Pintor et al., 1997c; 1999).

The experiments performed on human brain deserve special mention. Since healthy human brain tissue is not readily available, the number of these experiments has been limited. However, the answer to the main question of whether there are presynaptic diadenosine polyphosphate receptors has proved positive. As in the rat brain, in human brain synaptic terminals, dinucleotide receptors are coupled to N-type VDCC. This suggests new perspectives related to the possible therapeutic use of diadenosine polyphosphates and the antagonist Ip_5I, assuming we find dinucleotide receptors in terminals in which the release of a particular neurotransmitter is pathologically altered. The presence of the dinucleotide receptor in aminergic, cholinergic, GABAergic and glutamatergic synaptosomes and its effect on neurotransmitter release from these terminals have been recently explored in the rat brain (Giráldez et al., 2001; Gómez-Villafuertes et al., 2001; Díaz-Hernández et al., 2002b; *see* following sections).

The guinea-pig has been the model of choice to determine the distribution of the dinucleotide receptor in the brain areas: cortex, midbrain and cerebellum. Receptors fulfilling the requirements established for dinucleotide receptors were identified in the midbrain and cerebellum. In contrast, diadenosine polyphosphates and ATP appear to act via the same type of P2 receptor, presumably P2X, in guinea-pig cortical synaptosomes (Pintor et al., 1997c). Interestingly, the pharmacological profile of the dinucleotide receptor differs slightly according to the animal species.

Pharmacology of the Dinucleotide Receptor

Rat midbrain synaptosomes were used to analyze the pharmacology of the dinucleotide receptor by testing the effects of a series of diadenosine polyphosphates (Ap_nA, $n = 2$–5) along with the behavior of structural analogues of these substances such as oxidized-adenine dinucleotides ($o\text{-}Ap_nA$), etheno derivatives of diadenosine polyphosphates ($\varepsilon\text{-}Ap_nA$) and diguanosine polyphosphates (Gp_nG). All these compounds behaved as agonists of the dinucleotide receptor, but the responses differed according to the length of the phosphate chain. As a general rule, it was observed that dinucleoside polyphosphates with 2- and 5-phosphate bridges are full agonists at the dinucleotide receptor in rat brain synaptic terminals, while dinucleotides comprised of 3 or 4 phosphates behave as partial agonists (Pintor, Gómez-Villafuertes & Miras-Portugal, 2001). The reason for these differences remains unclear, though it might be that the charge distribution of the phosphate bridge generates different stacking conformations for each dinucleotide.

It is of note that despite the dinucleotide receptor being equally sensitive to both adenine and guanine dinucleotides, another group of compounds that contain hypoxanthine instead of adenine, the diinosine polyphosphates (Ip_nI), behaves as potent antagonists of the dinucleotide receptor, as mentioned previously. The structural differences needed to change the pharmacological properties of a dinucleotide from those of an agonist to an antagonist have yet to be established, but the antagonistic effect of Ip_nI is a starting point.

In guinea pig and mouse receptors, the situation changes. While in the rat, Ap_5A is the most effective diadenosine polyphosphate, other dinucleotides (Ap_4A and Ap_6A) are more effective than Ap_5A in the guinea-pig (Pintor et al., 1997c). For the receptor occurring in the mouse, Ap_6A is approximately twice as effective as equivalent concentrations of Ap_4A, and Ap_5A is less effective than Ap_4A (Pivorun & Nordone, 1996). This finding probably points to the existence of different subtypes of dinucleotide receptors. However, the presence of ectonucleotidases with different selectivity for the dinucleotides in the animal species under study could also account for the varying pharmacological profiles observed.

The scarcity of human brain tissue precludes the possibility of performing a complete pharmacological study, but results so far indicate possible discrepancies in the action of Ip_5I. Ca^{2+} increases induced by Ap_5A in human brain tissue were only partially blocked by this antagonist (Pintor et al., 1999) while it was able to completely block the response to dinucleotides in the

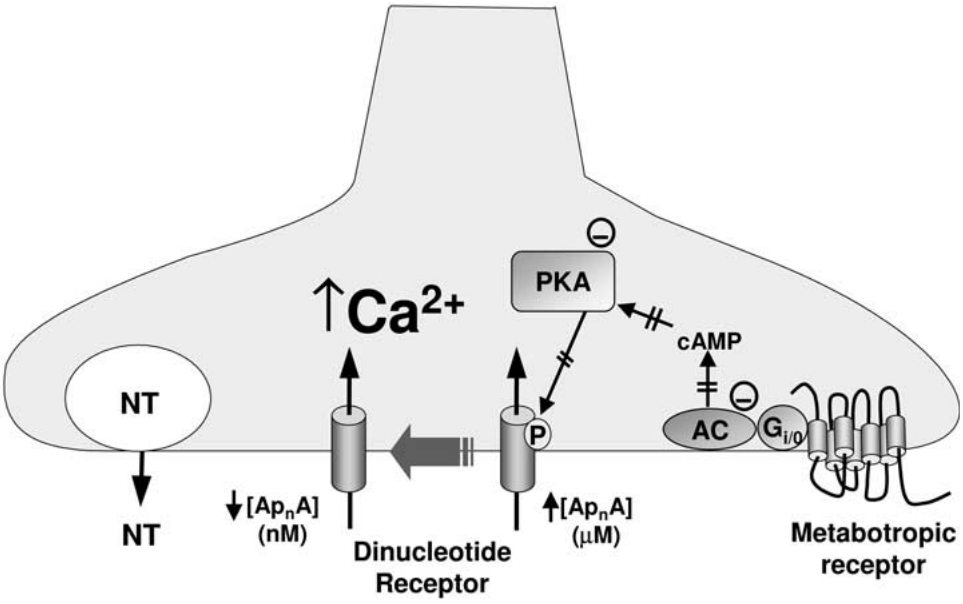

Fig. 1. Modulation of dinucleotide receptor activity. Diagram of dinucleotide receptor modulation achieved by stimulating second messenger systems negatively coupled to adenylate cyclase and by reducing PKA activity. The activation of presynaptic metabotropic receptors, such as adenosine A1 or $GABA_B$ receptors, allows the dinucleotide receptor to reach a higher affinity state, this receptor being sensitive to concentrations of diadenosine polyphosphates as low as in the nanomolar range.

rat brain model. This could suggest the human receptor is not as sensitive to the antagonist as that of the rat. Another possibility is that the human brain has a subpopulation of receptors sensitive to Ap_5A, which are not blocked by Ip_5I. The differences observed could, nevertheless, be explained by differences in dinucleotide-cleaving ectoenzymatic activities.

Modulation of Dinucleotide Receptor Activity

The dinucleotide receptor in rat midbrain synaptosomes is intensely modulated by protein kinases and phosphatases. Activation of PKA by forskolin (which activates adenylate cyclase (AC) and increases the levels of the physiological PKA activator, cAMP) leads to a drop in the Ca^{2+} transients induced by Ap_5A. The stimulation of PKC with phorbol esters such as 12,13-dibutyrate gives rise to a similar effect. Conversely, effectors that led to the inhibition of either PKA or PKC clearly potentiated the Ap_5A-induced Ca^{2+} responses in rat synaptosomes (Pintor et al., 1997b).

The possible involvement of a phosphorylation process in controlling dinucleotide receptor activity was further confirmed by analyzing the effects of different protein phosphatase inhibitors. These substances also produced a decrease in the calcium transients elicited by Ap_5A, indicating that several phosphatases, including protein phosphatase 2A and calcineurin, could reverse the phosphorylation achieved by PKA and PKC (Pintor et al., 1997b).

These results suggest the possibility that different agents acting on presynaptic receptors coupled to the activation of protein kinases and phosphatases could modify dinucleotide receptor activity. Indeed, modulation of the dinucleotide receptor by adenosine and ATP has been described in rat midbrain synaptosomes. ATP, acting on a not fully characterized P2 receptor, induces a decrease in the maximal response elicited by Ap_5A in these terminals. Adenosine, on the other hand, shows a dramatic effect on the affinity of the dinucleotide receptor for its ligand. This nucleoside, acting presumably on A1 receptors, allows the dinucleotide receptor to attain a high-affinity state, and thus to be stimulated by lower concentrations of diadenosine polyphosphates, even in the picomolar/low nanomolar range (Díaz-Hernández et al., 2000). It should be underscored that in the absence of A1 receptor activation, the dinucleotide receptor in rat midbrain synaptosomes is only sensitive to Ap_nA concentrations in the micromolar range (Pintor & Miras-Portugal, 1995; Pintor et al., 2001, *see* also preceding sections). Thus, the increased affinity induced by adenosine could confer the dinucleotide receptor the capacity to respond to more physiologically relevant Ap_nA concentrations, given the levels of these substances measured in perfusion samples from the caudate putamen of conscious rats using the in vivo push-pull perfusion technique (Pintor et al., 1993, 1995, *see* preceding section on Ap_nA release from neural models).

The ability of adenosine to induce a high-affinity state of the dinucleotide receptor has also been described in rat hippocampal nerve terminals. The activation of A_1 or A_{2A} receptors in these terminals caused a dramatic transformation of the sigmoid dose-re-

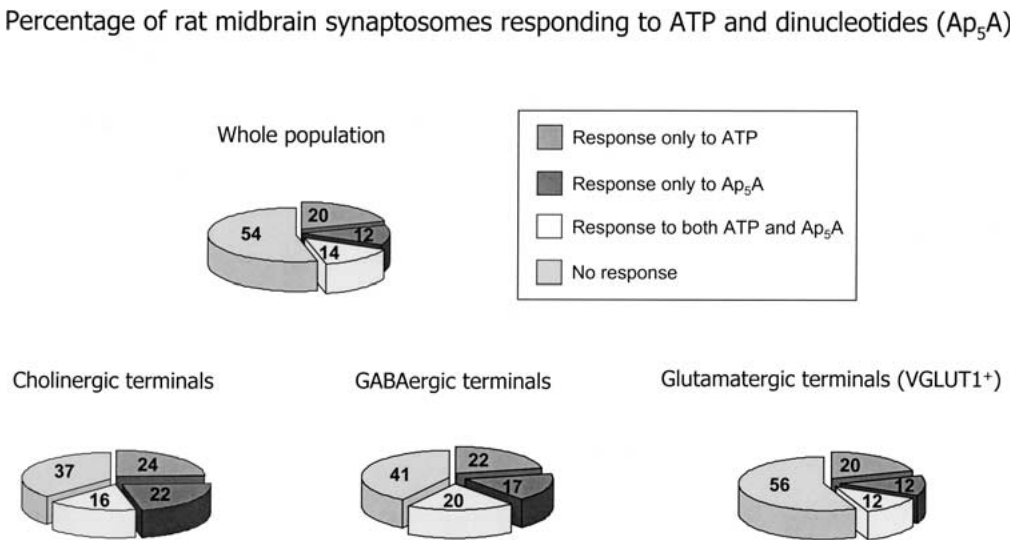

Fig. 2. Distribution of the responses to ATP and dinucleotides observed in cholinergic, GABAergic or glutamatergic midbrain synaptic terminals. Using antibodies against vesicular neurotransmitter transporters it is possible to characterize the different types of synaptic terminals in midbrain preparations. Thus, responses to the purinergic agents ATP and Ap_5A could be selectively analyzed in each type of terminal. The figure shows the proportions of synaptosomes responding to ATP, Ap_5A or both agonists, in cholinergic, GABAergic or glutamatergic midbrain terminals (the latter were identified using antibodies against VGLUT1). For comparative purposes, the response distribution of the entire synaptosomal population has been also included.

sponse curve for Ap_5A into a biphasic one with two clearly separated components. The new component of the Ap_5A dose-response curve showed an EC_{50} value in the picomolar/low nanomolar range. The second component showed an EC_{50} value in the low micromolar range, similar to those obtained for the Ap_5A dose-response curve in the absence of A_1 or A_{2A} agonists. Notably, activation of adenosine A_3 receptors in hippocampal terminals had the opposite effect, inducing a right-shift of the concentration-response curve for the dinucleotide. The physiological meaning of the different modulatory role of each adenosine receptor subtype is not known. However, since adenosine is the final product of extracellular catabolism of Ap_nA by ectonucleotidases, activation of adenosine A_1 and A_{2A} could be perceived as a feedback amplification loop to increase sensitivity to Ap_5A. In contrast, activation of A_3 receptors, achieved at higher adenosine concentrations, may represent a safety shut-down mechanism aimed at avoiding the excessive and uncontrolled functioning of this self-activating loop (Díaz-Hernández et al., 2002a).

Changes in dinucleotide receptor affinity have also been observed after the activation of presynaptic $GABA_B$ receptors (Fig. 1).

Dinucleotide Receptors in Single Synaptosomes

Two Independent Receptors for ATP and Ap_5A

By imaging intracellular Ca^{2+} in single synaptic terminals using the fluorescent dye fura-2AM in a microfluorimetric technique, it has been possible to gain detailed information indicating the existence of independent receptors for ATP and diadenosine polyphosphates, thus confirming the results derived from the synaptosomal populations.

In single isolated synaptic terminals studied by microfluorimetry, 100 μM ATP elicited a maximal Ca^{2+} increase of 217.4 ± 48.7 nM, while the same concentration of Ap_5A produced a 201.3 ± 50.3 nM increase. The presence of independent receptors was reconfirmed by analyzing individual terminals. Some terminals only responded to ATP challenges (20%), while others only reacted to Ap_5A (12%). A third population responded to both agonists (14%) (Díaz-Hernández et al., 2001). The existence of independent ATP and dinucleotide receptors was further confirmed using the antagonists PPADS and Ip_5I. Responses to ATP were abolished by pre-treatment with PPADS, but not by Ip_5I (a dinucleotide receptor antagonist in this model), whereas the Ca^{2+} transients elicited by Ap_5A were not blocked by PPADS but were abolished by Ip_5I (Díaz-Hernández et al., 2001). These results confirm our suggestion of independent ATP and diadenosine polyphosphate receptors based on observations made in synaptosomal population experiments (Pintor & Miras-Portugal, 1995).

Dinucleotide Receptors Are Coupled to VDCC

Dinucleotide receptor activation leads to a Ca^{2+} entry that is independent of calcium and sodium voltage-dependent channels. Nonetheless, it should be noted that this initial cation influx produces a depolarization threshold which activates both sodium and calcium

voltage-dependent ion channels (Pintor & Miras-Portugal, 1995).

Once again, this point was reconfirmed by the single synaptosome imaging technique. The binomial nature of dinucleotide receptor/calcium channels can be demonstrated by pre-treating the synaptic terminals with cadmium (Cd^{2+}). Individual terminals containing dinucleotide receptors severely reduced their Ca^{2+} influx in the presence of Cd^{2+} in the medium, after challenge with Ap_5A. Despite this reduction, there was always a measurable amount of cations entering the terminals, presumably through the dinucleotide receptor. This was confirmed by removing the extrasynaptosomal Ca^{2+} source, which led to the complete inhibition of the response to Ap_5A (Díaz-Hernández et al., 2001, 2002b).

A more in-depth study on voltage-dependent ion channels involved in the Ap_5A-evoked Ca^{2+} increase was performed using more specific pharmacological tools. Dihydropyridines, and toxins such as agatoxins and conotoxins, are useful tools to investigate the part played by L-, N- and P-type voltage-dependent calcium channels in a large variety of neural preparations (Meir et al., 1999). When terminals are pre-incubated with the N-type voltage-dependent calcium channel blocker ω-conotoxin G-VI-A, the response induced by Ap_5A is reduced by about 60%. In addition, the P/Q-type voltage-dependent channel blocker ω-conotoxin M-VII-C, was able to reduce the response elicited by Ap_5A roughly to the extent of 40%.

Other channels such as L-type or T-type were not involved in the activity of Ap_5A, since no modification was achieved by either nicardipine or Ni^{2+} (Díaz-Hernández et al., 2001). It may be concluded that in isolated rat midbrain synaptic terminals, after an initial Ap_5A-elicited Ca^{2+} influx, a depolarization threshold triggers the opening of N-type and P/Q-type voltage-dependent calcium channels, leading to more intense depolarization, which could, in turn, induce the release of neurotransmitter.

Diadenosine Polyphosphates Induce Neurotransmitter Release

It seems reasonable to propose that the Ca^{2+}-entry elicited by diadenosine polyphosphates also involving VDCC activation, may induce the release of the neurotransmitters present in synaptosomal synaptic terminals. We explored this interaction between dinucleotide receptor activation and the exocytotic release of other neurotransmitters.

Dinucleotide Receptors in Cholinergic Terminals

The existence of presynaptic nicotinic receptors at the CNS is now widely accepted. Activation of these receptors, basically comprised of α (mainly α3, α4 and α7) and β subunits, allows Ca^{2+} entry into the synaptic terminals (Wonnancott, 1997).

The presence of dinucleotide receptors in rat midbrain cholinergic terminals has been demonstrated by immunocytochemical methods and Ca^{2+} measurements. Markers such as the anti-vesicular acetylcholine transporter (VAT) were used to immunolocate cholinergic terminals and allowed to show that some of these terminals have Ca^{2+}-responding diadenosine polyphosphate receptors (Díaz-Hernández et al., 2002b).

Once again, and as previously described, cholinergic terminals presented three terminal types, one type that responded only to Ap_5A (22.4%), another type responding to ATP (24.7%) and a third population sensitive to both compounds (16.3%). These cholinergic terminals responded to Ap_5A only in the presence of extracellular Ca^{2+}. Moreover, when the cholinergic terminals were pre-incubated with Cd^{2+}, the Ca^{2+} transients elicited by the dinucleotide were severely diminished by almost 45% (Díaz-Hernández et al., 2002b).

One of the most interesting issues related to finding dinucleotide receptors in cholinergic terminals would be to establish whether or not the Ca^{2+} influx elicited by Ap_5A would be sufficient to trigger acetylcholine release. In a series of experiments conducted using Israel and Lesbats' (1981) chemoluminescent technique, we demonstrated that both ATP and Ap_5A could induce the exocytotic release of acetylcholine in a dose-dependent fashion. For Ap_5A, the EC_{50} value was 1.5 μM and its maximal effect was 46.8 pmol/mg protein, which was slightly higher than the effect induced by ATP (34.1 pmol/mg protein). Neither Ap_5A nor ATP were able to induce the release of acetylcholine in the absence of extracellular calcium or in the presence of their respective antagonists (Ip_5I for Ap_5A and PPADS for ATP).

These cholinergic terminals also have nicotinic receptors. Nicotine showed a concentration-dependent response pattern with an EC_{50} value of 7.91 μM. Moreover, Cd^{2+} similarly caused a 48.2% reduction in the response induced by nicotine. Regarding the colocalization of nicotinic and dinucleotide receptors in these cholinergic terminals, it was: 7.6% of terminals responding to nicotine and Ap_5A; 11.5% responding to nicotine, epibatidine and Ap_5A; and 7.6% responding to Ap_5A alone (Díaz-Hernández et al., 2002b).

Dinucleotide Receptors in Aminergic Terminals

Aminergic terminals are very abundant in basal ganglia nerve endings. In isolated synaptic terminals obtained from this region of the rat brain, the presence of receptors for diadenosine polyphosphates

coupled to a Ca^{2+} increase was demonstrated (Giraldez et al., 2001). Using antibodies raised against vesicular monoamine transporter 2 (VMAT2), it has been possible to immunolocalize monoaminergic terminals. Three population types were again revealed: a type responding only to Ap_5A (16.9%), another type responding only to ATP (8.2%) and a third type responding to both (32.6%). Terminals challenged with Ap_5A responded to this dinucleotide by increasing their intrasynaptosomal Ca^{2+} concentration in a similar way as for the other terminals already described (Giráldez et al., 2001).

Dinucleotide Receptors in Aminoacidergic Terminals

The co-storage and co-release of nucleotides such as ATP and GABA has been demonstrated in rat spinal cord dorsal horn neurons (Jo & Schlichter, 1999). In this model as well as in hippocampal neurons, ATP facilitates GABA release by activating presynaptic P2X receptors (Inoue, 1999; Hugel & Schlichter, 2000). Only recently has it been possible to evaluate the connection between diadenosine polyphosphates and GABA.

GABAergic rat midbrain synaptic terminals can be easily identified using antibodies against glutamic acid decarboxylase (GAD) and vesicular inhibitory aminoacid transporter (VIAAT). Synaptosomes, characterized as GABAergic terminals, responded to ATP and Ap_5A, producing intrasynaptosomal Ca^{2+} increases. As described in the previous paragraphs, three populations of GABAergic terminals were found according to their response to ATP and Ap_5A. Some terminals only responded to Ap_5A (17%), others only to ATP (22%) and a third population responded to both agonists (20%). The applications of either ATP or Ap_5A to GABAergic terminals induced the release of GABA in a dose-dependent way. The EC_{50} value for Ap_5A was 19.5 μM and the maximal secretory effect for this dinucleotide was 97 pmol/mg protein. The effect of Ap_5A was dependent on extracellular Ca^{2+} levels and also sensitive to the dinucleotide receptor antagonist Ip_5I (Gómez-Villafuertes et al., 2001).

Glutamatergic terminals can be clearly identified by the presence of the vesicular glutamate transporters (VGLUT1 or VGLUT2). Again, different populations of these terminals could be observed on the basis of their response to ATP and Ap_5A (Fig. 2). Stimulation of midbrain glutamatergic terminals with Ap_5A also induced glutamate release. This release was dependent on the concentration of the agonist.

Conclusions

The presence of dinucleotides in secretory granules and their exocytotic release indicates an extracellular role for these compounds. The presence of specific ionotropic receptors for dinucleotides demonstrated in synaptosomal populations confirms their signalling role. At this level, they are able to induce a calcium influx that can lead to the exocytotic release of stored classical neurotransmitters such as acetylcholine, GABA or glutamate.

The presynaptic dinucleotide receptor appears to be strictly regulated by the actions of protein kinases and phosphatases, originally under the control of presynaptic metabotropic receptors. Activation of G_i/G_o-coupled receptors results in a significant increase in the affinity shown by the dinucleotide receptor.

The findings of experiments performed on individual synaptic terminals have much improved our knowledge on the signalling activity of dinucleotides. These experiments have served to confirm the existence of specific receptors for Ap_nA, independent of receptors responding to ATP. In many cases, these receptor types were located in different nerve terminals. Moreover, these experiments confirmed that calcium entry through the dinucleotide receptor is capable of inducing the further opening of N- and P/Q-type voltage-dependent calcium channels.

Evaluations made of the distribution of the presynaptic dinucleotide receptor within the different neurotransmitter-containing terminals reveal their abundance in cholinergic and GABAergic terminals, exceeding proportions detected in the entire synaptosomal population.

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